

Quantitative, Comparable Hyperspectral Chemical Imaging of Biological Specimens with Broadband Coherent Anti-Stokes Raman Scattering (BCARS) Microspectroscopy

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Abstract— Coherent Raman imaging (CRI) methods probe the intrinsic molecular vibrations of chemical species, providing a unique window into chemical composition and state without the addition of exogenous labels or stains. These methods interrogate the composition of cell populations or tissue specimens, potentially linking such factors as chemical composition and cell function or pathology. A significant challenge to harnessing the rich chemical content of CRI spectra is the separation of the chemically-sensitive Raman vibrational content from a relatively chemically-insensitive background, the so-called “nonresonant background” (NRB). Through coherent mixing, the NRB amplifies the weak Raman signal but in turn distorts the spectral lineshapes. Developments in “phase retrieval” techniques have demonstrated removal of the NRB under ideal conditions, but in reality may result in significant phase and amplitude errors that until now have been neglected. In this work, we demonstrate from physical principles the provenance and rectification of these errors. This pre-processing method most importantly generates spectra that are microscope system agnostic and removes many experimental factors; thus, spectra are quantitatively comparable within and between samples regardless of operating conditions.

Index Terms—Coherent anti-Stokes Raman scattering (CARS), phase retrieval, quantitative analysis.

I. INTRODUCTION

Traditional microscopy modalities enable chemical sensitivity and specificity through exogenous stains or fluorophores. Although a powerful technique, these methods require, to varying degrees, *a priori* knowledge of sample composition and targets of interest. Additionally, these methods may be destructive or impede normal physiological operation of live specimens. Thirdly, these techniques practically have a limitation on the number of “colors” that can be measured simultaneously. Fluorescent labels, for example, have broad emissions spectra; thus, the use of more than 3 or 4 fluorophores (typically) creates significant challenges in distinguishing one color from another. Label-free technologies,

on the other hand, probe intrinsic properties of the sample. Vibrational spectroscopies, such as infrared absorption and Raman scattering microspectroscopies, interrogate molecular vibrations that collectively provide a view into the sample composition, local conformation, and thermodynamic state. These methods require no *a priori* knowledge, and vibrational spectra may contain over a thousand colors, which greatly expands when one considers combinations/permutations of the individual colors. Traditional methods, however, are often hampered by lengthy acquisition times or destructive sample preparation, such as dehydration to remove the spectral influence of water. Recently, there has been interest in high-speed vibrational spectroscopies, most notably coherent anti-Stokes Raman scattering (CARS), that take advantage of nonlinear optical scattering to generate spectra at significantly higher rates than the linear methods.

CARS is a nonlinear phenomenon in which a “pump” and a “Stokes” photon coherently excite a molecular vibration from which a “probe” photon can inelastically scatter [1]. Through multiple stimulation-scatter events of multiple vibrational modes, a spectrum can be recorded. In comparison to traditional [spontaneous] Raman scattering, this coherent stimulation not only enhances the rate of vibrational excitation, but also directs the scattered photons in the forward and backward direction, which enables significantly more efficient collection than with isotropic radiation [2]. Additionally, the photons are blue-shifted from the excitation sources; thus, not competing with autofluorescence. CARS, and related techniques, have demonstrated success in biological [3] and materials imaging [4], primarily presenting pseudocolor imagery from which biological conclusions may be drawn. Taking full advantage of the rich detail provided through CARS spectra, however, has been significantly hampered due to the distorting influence of the NRB.

Although the CARS signal does not overlap with autofluorescence, other nonlinear optical phenomena,

predominantly electronic in nature, generate an overlapping signal, collectively known as the “nonresonant background” (NRB). So ubiquitous is the NRB that the term “CARS signal/spectrum” implies a mixed Raman-NRB signal. Although historically maligned, the NRB actually acts as a strong heterodyne amplifier, boosting the weak Raman signal above the noise floor. Much effort has been applied to physically reducing the generation of the NRB, but this has primarily reduced the Raman signal to unacceptably low levels. Alternatively, numerical methods have developed to perform phase retrieval that effectively enables separation of the Raman and NRB signal components [5,6]. These methods have demonstrated success but work under the strict assumption that the NRB and optical system response are directly measurable. As the NRB is not practically measurable in isolation of the vibrational response, surrogate materials are used that [ideally] contain no Raman peaks with the assumption that the NRB is completely chemically insensitive. This assumption leads to amplitude and phase errors that are sample, surrogate, and system specific, which significantly hinders reliable quantitative analysis and robust biological findings.

We have re-developed phase retrieval in view of the practical use of surrogate NRB materials [7]. Through a theoretical analysis of generated errors, we have revealed that the phase and amplitude errors are identifiable and removable in post-processing without changes to the typical workflow. Using biological tissue, we experimentally demonstrate a significant improvement in spectral consistency regardless of microscope system and surrogate NRB utilized.

II. THEORY

The generation of the frequency-domain (ω) CARS signal, I_{CARS} , may be described mathematically as:

$$I_{\text{CARS}}(\omega) \propto \left| \left\{ \chi^{(3)}(\omega) [I_{\text{Stokes}}(\omega) \star I_{\text{pump}}(\omega)] \right\} \star I_{\text{probe}}(\omega) \right|^2 \quad (1)$$

where I_{Stokes} , I_{pump} , and I_{probe} are the Stokes, pump, and probe sources, respectively, $\chi^{(3)}$ is the 3rd-order nonlinear susceptibility that describes the material response (Raman vibrational and NRB), and ‘ \star ’ and ‘ \star ’ are the cross-correlation and convolution operations, respectively. The cross-correlation of the pump and Stokes sources represents the stimulation profile, which determines the intensity and bandwidth of excitation, and the probe source bandwidth determines the system spectral resolution. The nonlinear susceptibility may be expanded as a summation of Raman components, χ_{R} , and “nonresonant” components, χ_{NR} , that contribute to the generation of the NRB. Under infrared excitation, as is typically employed in CARS systems, χ_{NR} is approximately real as there are typically no electronic resonances. Additionally, for simplification in this work, we will consider a probe source that is spectrally narrow with respect to the Raman lineshapes; thus, $I_{\text{probe}}(\omega) \approx I_{\text{probe}} \delta(\omega)$, where $\delta(\omega)$ is a delta-function (unit impulse response).

To a first-degree approximation, the imaginary portion of χ_{R} is proportional to the spontaneous Raman response. As the CARS signal is proportional to the modulus of the total nonlinear susceptibility, the purpose of phase retrieval techniques is to ascertain a phase, $\phi(\omega)$, that allows a separation of the Raman and NRB terms. Previous research demonstrated that if one could measure the NRB, $I_{\text{NRB}}(\omega)$, independently, a phase could be extracted using the Kramers-Kronig relation (i.e., through a Hilbert transform, \hat{H}) [5,7]:

$$\phi(\omega) = \hat{H} \left\{ \frac{1}{2} \ln \frac{I_{\text{CARS}}(\omega)}{I_{\text{NRB}}(\omega)} \right\} \quad (2)$$

From this equation, the retrieved “Raman-like” spectrum, $I_{\text{RL}}(\omega)$, is calculated from:

$$I_{\text{RL}}(\omega) = \sqrt{\frac{I_{\text{CARS}}(\omega)}{I_{\text{NRB}}(\omega)}} \sin \phi(\omega). \quad (3)$$

As previously mentioned, however, $I_{\text{NRB}}(\omega)$ is not directly measurable; thus, a reference/surrogate spectrum $I_{\text{ref}}(\omega)$ is utilized. The result is a Raman-like spectrum that appears to lie on top of a slowly-varying background. Looks, however, can be deceiving. As can be shown [7], the retrieved spectrum is not simply the Raman-like spectrum with an additive error. Rather, the use of $I_{\text{ref}}(\omega)$ causes phase and amplitude errors, with the phase error additive and the amplitude error multiplicative.

III. MATERIALS AND METHODS

Broadband CARS (BCARS) spectra and images are collected on a recently-developed microscope platform with a unique stimulation paradigm for significantly enhanced signal generation [3]. Raman spectra were extracted and error-corrected in MATLAB using a re-developed implementation of the Kramers-Kronig relation [7].

IV. RESULTS

Figure 1 compares the use of traditional processing with the newly developed phase retrieval and error correction method. Specifically, Fig. 1 (a) shows a pseudocolor image of mouse skin, highlighting lipids in red (2850 cm⁻¹), collagen fibers in green (855 cm⁻¹), and DNA/RNA in blue (785 cm⁻¹). The Raman-like spectra were extracted from the raw BCARS spectra via the Kramers-Kronig relation and any baseline was subtracted using an asymmetric/penalized least square fitting algorithm [8,9]. The left half of Fig. 1 (a) was processed using water as the NRB spectrum and the right half using coverslip glass. There is a clear distinction between the two halves of the image with the DNA/RNA and collagen being clearly brighter when using water. From a single-point spectrum from a sebaceous gland, as shown in Fig 1 (c), one can see the lipid spectrum is conversely stronger when using glass as the NRB reference. Comparison of these two spectra demonstrate that the phase and amplitude errors in phase retrieval lead to intra-

spectral distortions of the Raman-like spectrum that cannot be simply normalized out.

In contrast, Fig. 1 (b) shows the pseudocolor image of the same murine skin with the same contrasts but using the newly developed phase retrieval and error correction method. With the new method, there is no clear distinction between the two halves of the image. Figure 1 (d) shows the single-pixel spectra from a sebaceous gland (same spatial point as in Fig. 1(c)) showing very close agreement.

V. CONCLUSION

We have developed a new phase retrieval and error correction method that produces spectra that are CARS microscope (spectrometer)-independent and mitigates the effect of using surrogate NRB spectra. This method does not change the experimental workflow of CARS imaging and is performed *in silico*. These efforts will significantly improve the reliability and robustness of biological findings using CARS spectroscopy as this facilitates direct comparison of spectra collected on any CARS system. Additionally, this method promotes a change in data “currency” for hyperspectral CARS imagery as it is now meaningful to disseminate entire datasets for community examination and analysis in addition to pseudocolor images.

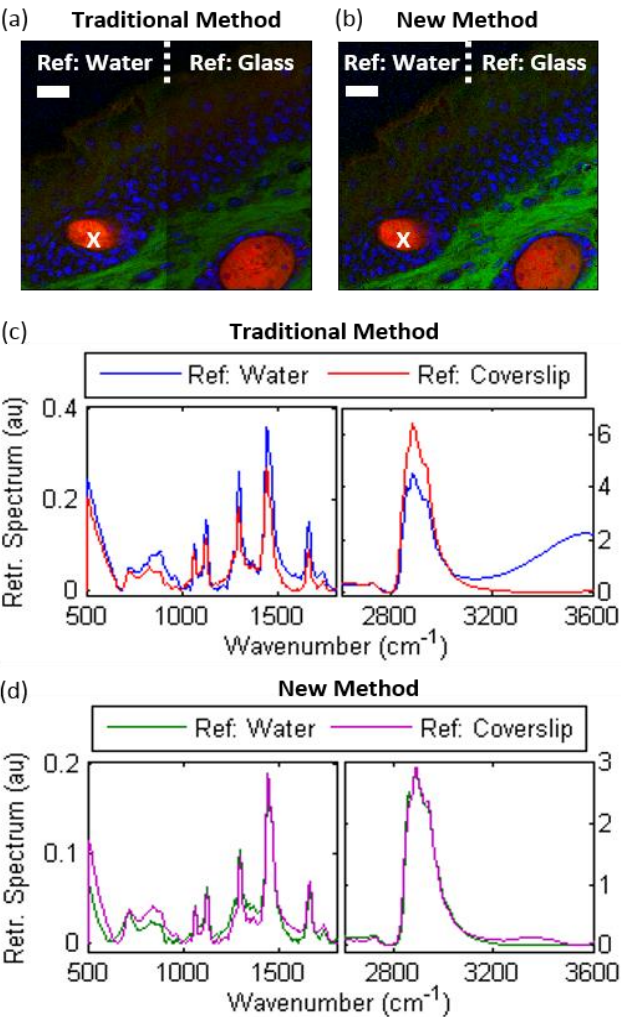


Figure 1: (a) BCARS image of murine skin highlighting lipids in red, collagen fibers in green, and DNA/RNA in blue processed using traditional phase retrieval with water and glass coverslip spectra as a surrogate for the NRB. (b) Same images as (a) using the newly-developed phase retrieval and error correction method. Single-pixel spectra from a sebaceous gland [marked with an ‘x’ in (a,b)] using traditional (c) and the new (d) processing methods.

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